EXPERIMENTAL STUDIES ON THE CAUSES OF REFLUX ESOPHAGITIS, WITH ESPECIAL EMPHASIS ON THE SIGNIFICANCE OF ESOPHAGEAL CATHEPTASE, BILE AND BACTERIAL INFECTION

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by

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(Received for publication Jun. 10, 1959)

FOREWORD

Keeping pace with improvements brought in the technique and method of surgical operation, progress in antibiotic substances, advancement in the method of anesthesia, blood transfusion and nutritional treatment before and after a surgical operation as well as in measures to prevent development of complications following an operation, the esophagus surgery has made remarkable progress in recent years, and its operative mortality has steadily been reduced.

It is reported that, when the esophagus is anastomosed to the stomach, duodenum or jejunum, following the resection of esophago-gastric junction and total gastrectomy, or when cardioplasty is executed against cardiospasm, a considerable high percentage of reflux esophagitis will result, leading to frequent postoperative complaints.

Regarding the causes of reflux esophagitis, a number of different theories have been advanced. Such causes as the action of digestive juice, the damage due to acid and alkali, bacterial infection and the interruption of local circulation, were given importance.

TAKATSUKI, one of our co-workers, undertook to manufacture the purified powders of mucous protein from various parts of the digestive tract, and, by comparing quantitatively their respective resistance to protease activity, substantially established that the esophageal mucous protein is provided with the lowest resistance among various parts of the digestive tract against the digestive activity by pepsin and trypsin.

He also established in perfusion experiments in the esophagus of dogs, that, by causing a local interruption of blood supply, or by adding cysteine, the activating substance of cathepsin to perfusing solution, that the degree of damage to the esophageal mucous membrane is perceptibly heightened. It was also noticed that, the destruction of mucous membrane composition by trypsin was increased by adding bile to the perfusing solution.

The author, especially with an eye on the fact that reflux esophagitis is liable to be caused near the anastomosed portion, where the esophagus was devascularized, and that the living esophageal mucous proteine is hardly digested by pepsin or

trypsin, has undertaken a chemical examination about internal factors, beginning with the esophageal cathepsin, and, again, about the significance of the bile and, bacterial protease mixed in the contents of the reflux.

PART I. ON THE SIGNIFICANCE OF THE INTERRUPTION OF CIRCULATION AND ESOPHAGEAL CATHEPSIN IN THE OCCURRENCE OF REFLUX ESOPHAGITIS

Chapter 1. On the Quantity of Blood Supply in Various Parts of Normal Esophagus and Changes of the Quantity of Blood Circulation as a Result of the Isolation of Esophagus.

Section 1. Arteries Distributed in Esophagus of Experimental Dogs.

Many reports have so far been made about the arteries distributed in the esophagus. In Japan, OGAI reported on the experiment in rabbits, dogs and human beings in 1932, while IPPONSUGI, in 1955, conducted studies of the system of blood vessel distribution in esophagus, and also, of its microscopic composition. The author has likewise carried out a series of examinations on dogs. In these examinations, physiological salt solution was injected into the thoracic aorta, and following perfusion, gelatin-added India ink, radiopaque mass, or synthetic resin was injected. The overall result obtained ascertained to coincide well with the results so far obtained by his predecessors (Figure 1). Namely, the main arteries distributed in the cervic esophagus proved to be the tracheoesophageal branches of Inf. Thyroid Arteries (A. thyreoidea inf.) and Sup. Thyroid Arteries (A. thyreoidea sup.).

The main arteries distributed in the thoracic esophagus were found to be esophageal branches starting from the Aa. bronchiales, both on the front and back sides. On the back side, certain Aa. oesophagei propriae existed. The main arteries distributed in the abdominal esophagus were the esophageal branches of A. gast. sinist. and a branch of A. diaphragmatica.

Section 2. Experiment on the Isolation of Thoracic Esophagus.

Wound healing of a suture executed on the esophageal tissue has hitherto been considered to be difficult, and one of the principal reasons for it was considered to exist in the fact that the esophageal blood vessels, unlike those of stomach or intestines, are poorly distributed and blood circulation is liable to be interrupted at the time of the isolation or movement of the esophagus.

However, PARKER, MACMANUS and others maintain that necrosis of the esophagus will never be caused by an interruption of esophageal blood circulation. The author, likewise, has carried out an experiment on the isolation of the esophagus to examine whether the esophagus is more liable to cause necrosis as compared with other digestive tracts.

(1) Method of Experiment:

Four adult mongrel dogs ranging in weight from 7 to 10 kg were used. Operations were performed under intravenously administered mintal anesthesia. With the air tract maintained by means of a domestically-made closed-circulation-typed intratracheal anesthesizer, and with oxygen inhalation undertaken, pressure

was applied and the right chest was opened, and the entire length of the thoracic esophagus was completely isolated from the adjacent tissues (Figure 2). The esophagus of two dogs were divided at a certain height. And then the anastomosis was performed in two layers with silk thread. In the course of the operation, no blood transfusion was undertaken, while the bleeding was limited to a minimum amount. After the operation, penicillin (crystal) 200,000 units, and streptomycin 0.5 g were injected into the thoracic cavity.

Table 1 Devascularization of the thoracic esophagus.

Dog No.	Sex	Body weight	Anastomosis	Length of survival	Cause of death
23	♂	6.0	None	1	Shock
24	♂	25.0	None	10	Emaciation
26	♀	8.0	Lower thirds	3	Sacrificed
28	♂	10.0	Lower thirds	5	Sacrificed

(2) Experimental Result:

The results of this experiment are shown in Table 1 and Figure 3. It is established that, even when the entire length of the thoracic esophagus is isolated from the adjacent tissues, no necrosis will occur in any part of the esophagus. In histological observation (Fig. 4), the esophagus was found to be in a perfectly normal condition, no abnormality being observed.

Section 3. Measurement of Amount of Circulating Blood in Normal and Isolated Esophagus, by Means of Measurement of Quantity of Distributed P^{32} Labeled Erythrocytes.

Ogai has reported on the distribution of blood vessels in the esophageal wall by means of the determination of quicksilver. Earlier, Majima, our collaborator, measured the amount of circulating blood in the normal esophagus by using P^{32} labeled erythrocytes, and obtained a result approximately similar to that obtained by Ogai.

(1) Method of Experiment:

According to Majima's method, as described in the preceding paper, P^{32} labeled erythrocytes, 80 cc, was injected intravenously into the dogs, whose thoracic esophagus was isolated from the adjacent tissues, and, 30 minutes afterwards, these dogs were sacrificed. Their esophagus, then was cut into many small pieces, and reduced to ashes by dry incineration in an electric kiln. By using a Geiger counter, the count value at each region of the esophagus was measured per unit weight.

(2) Experimental Result:

In the normal esophagus, as shown in Table 2 and Figure 5, the amount of circulating blood was smaller as compared with other portions of the digestive tract, showing an especially low value in the lower part of the thoracic esophagus. Again, on the occasion of the isolation of the esophagus, the value lowered to about 50% of the average count value of the stomach or small intestines.

Chapter 2. Quantitative Study of Distribution of Cathepsin in Esophagus and Other Parts of Digestive Tract.

Table 2 Measurement of amount of circulating blood in isolated esophagus by using of P^{32} labeled erythrocytes.

	Weight of slice (g)	cpm (c)	c—nc	c—nc/w	%
1	1.22	966	904	741	71.9
2	1.00	586	524	524	50.9
3	1.28	687	625	488	47.4
4	1.09	719	657	603	58.5
5	1.00	676	614	614	59.6
6	1.19	867	805	676	65.6
7	1.33	1030	968	727	70.6
8	1.30	724	662	509	47.5
9	1.30	873	811	624	60.6
10	1.30	1153	1091	840	81.5
11	1.10	1201	1139	1035	100.5
12	1.40	1386	1324	946	
13	1.32	1334	1272	964	mean 1030
14	1.10	1315	1253	1139	100.0
15	1.20	1345	1283	1069	

Cathepsin is an enzyme named so by WILLSTÄTTER R. & E. BAMANN in 1929, which, decomposes undissociated auto-tissue protein on the isoelectric point and functions decomposingly in the weak-acid, reducing system, but, in the alkali, oxidizing system, functions synthetically. It is activated by H_2S, cysteine and is liable to be inhibited by halogen compounds, and is especially related to autolysis, necrosis and regeneration.

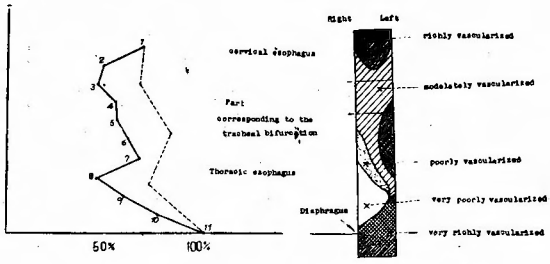

Fig. 5 Distribution of the P^{32} labeled erythrocytes at each region of the esophagus.
····· After the devascularization.
—— Before the devascularization.

(1) Method of Experiment:

(i) Enzyme solution: The enzyme material was prepared according to the UCHINO's method of producing catheptase non-containing ereptase. The mucous layer, muscular layer or a tunica serosa were taken out of various parts of the digestive tract. Each of these then, was added 1.5 volume of water ground with an homogenizer. The homogenate was made into pH 4.0 with 3% hydrochloric acid, incubated for 40 minutes at 37°C, and dried up to make an acetone-ether dried powder. This 10% solution in distilled water was made into an enzyme source. A fresh material was used, and by using ice and dry ice, degeneration of the enzyme material was prevented.

(ii) Substrate solution: A pure gelatine was used by dissolving it in a buffer solution at the rate of 4%.

(iii) Buffer solution: McILVAINE's citrate buffer solution (pH 4.0-4.5) was

used.

(iv) Method of measurement: As the principal reaction composition, enzyme solution 2.0 c c, 0.05 M cysteine hydrochloride solution (neutralized by adding NaOH solution) 2.0 c c, 4% gelatine buffer solution 10.0 c c and toluene 1.0 c c were mixed, after which pH was rectified, which was incubated for a certain period (24-48 hours) at 37°C, and, 5.0 c c of the reaction composition was taken with pipette before and after reaction, to which 1 ‰ phenolphthalein was added as an indicator, and, according to SOERENSEN's formol-titration, its acidity was measured. The acidity increase during reaction corresponded to the decomposed degree of substrate by means of the enzyme solution. As control, the acidity increase due to self-digestion of enzyme and the substrate itself was measured and rectified.

(2) Experimental Result:

(i) The optimal pH of cathepsin distributed in the esophageal mucosa existed between pH 4.5-5.0 (Table 3, Figure 6).

Table 3 Optimal pH of esophageal catheptic activity.
Acidity increase (N/10 NaOH c c) 48 hrs

pH 4.0	0.04
4.5	0.06
5.0	0.06
6.0	0.00
7.0	0.02

(ii) Activation of catheptic activity of esophageal mucosa by cysteine (Table 4, Figure 7): Activation was noticed to be most manifest by adding cysteine hydrochloride of 0.05 M at a density.

(iii) Amount of cathepsin distributed in various parts of digestive tract (Tables 5-8, Figure 8): All of healthy and adult mongrel dogs were sacrificed. The digestive tract from esophagus to colon, was taken out and various regions of the digestive tract were dissected layer by layer. Their catheptic activities were measured.

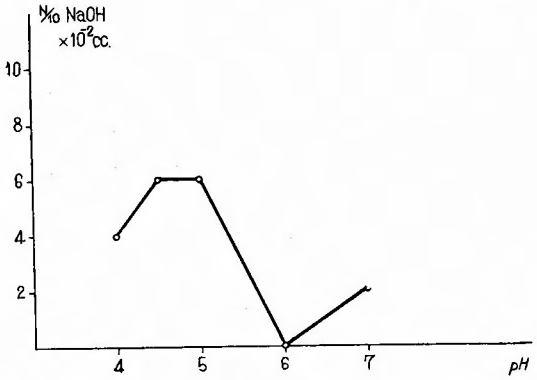

Fig. 6 Optimal pH of esophageal catheptic activity.

Table 4 Activating effect of cystein on the esophageal cathepsin.
Acidity increase (N/10 NaOH c c) 24 hrs

Concent. of cystein	Gelatine		
	pH	Esophagus	Liver
Control	4.5	0.02	0.39
0.05 M	4.5	0.06	0.80
20mg/20cc	4.5	0.06	0.78
10mg/20cc	4.5	0.02	0.70

In each region, cathepsin was seen to be distributed in large amount in the

Table 5 Distribution of cathepsin in the digestive tract.
Acidity increase (N/10 NaOH c c) 24 hrs

Organ		pH 4.0				pH 4.5	
Esophagus	M	0.04	0.04	0.02		0.03	0.06
	S	-0.07	0.01	0.00		0.04	0.04
Stomach	M	0.12	0.12	0.16	0.13	0.23	0.26
	S	0.03	0.00	0.03		0.07	0.03
Small intestine	M	0.62	0.73	0.65			0.29
	S	0.00	0.03	0.02			0.17
Colon	M	0.08	0.05	0.09			0.05
	S	0.00	0.00	0.00			0.06
Liver			0.84			0.72	

M : Mucous layer
S : Muscular or sero-muscular layer

Table 6 Distribution of cathepsin in the digestive tract of dogs.
Acidity increase (N/10 NaOH c c) 48 hrs

Organ		pH 4.0		
		i	ii	iii
Esophagus	M	0.05	0.07	0.03
	S	0.02	0.02	0.03
Stomach	M	0.15	0.19	0.25
	S	0.05	0.02	0.04
Small intestine	M	0.72	0.90	0.41
	S	-0.02	0.04	0.12
Colon	M	0.18	0.09	0.12
	S	0.08	0.04	-0.05
Liver			1.01	

mucous layer. Classified into each region, the amount of distribution was found to decrease gradually in the small intestines, stomach, colon and esophagus, in the order mentioned.

(iv) Amount of cathepsin contained in necrotic region of small intestines (Table 9) : As in the experiments carried out by MAJIMA and TSUKUDA, the jejunum was transplanted antethoracically (the length of the part of jejunum, transplanted outside the abdominal cavity was 40 cm). 48 hours later, the catheptic activity in the part affected with necrosis was compared with the healthy part for measuring.

Table 7 Distribution of cathepsin in the digestive tract of dogs.
Acidity increase (N/10 NaOH c c)

Organ		24 (hrs)		48 (hrs)
		pH 4.0	pH 4.5	pH 4.0
Esophagus	M	0.03	0.05	0.06
	S	0.00	0.04	0.02
Stomach	M	0.12	0.27	0.20
	S	0.01	0.05	0.04
Small intestine	M	0.65	(0.30)	0.70
	S	0.02	0.17	0.02
Colon	M	0.08	0.05	0.13
	S	0.00	0.06	0.05
Liver		0.78		1.01

Table 8 Distribution of cathepsin in the digestive tract of dogs.
Acidity increase (N/10 NaOH c c) 24 hrs

Organ		pH 4.5	
		M	S
Stomach	Cardia	0.25	0.03
	Pylorus	0.27	
Small Intestine	Duodenum	0.28	0.16
	Jejunum and Ileum	0.29	0.03
Esophagus		0.06	0.04
Appendix		0.06	0.05
Colon		0.05	0.06
Liver		(0.69)	
Necrotic portion of the stomach		0.04	0.03

The lower part of this Table coincided with the bordering part of necrosis, but, for the measurement of enzyme activity by means of formol-titration, a big amount of enzyme material was required, and so, the bordering region with the small portion of the necrotic region and normal region was simultaneously measured as the middle portion. While the necrotic region was found to carry a small amount of distributed cathepsin as compared with the normal region, the result obtained amply indicated that the amount of cathepsin was abundant in the bordering region.

PART II. ON THE SIGNIFICANCE OF BILE AND BACTERIAL PROTEASE IN THE OCCURRENCE OF REFLUX ESOPHAGITIS

That bile must play an important role in the occurrence of reflux esophagitis

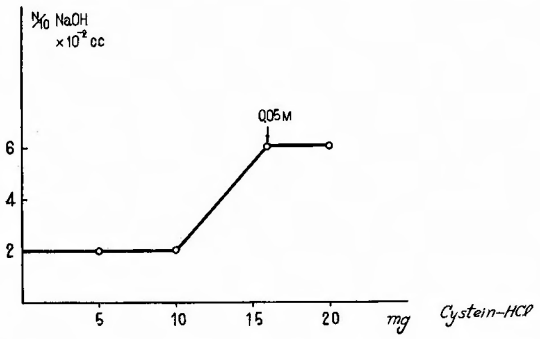

Fig. 7 Activating effect of cystein on the esophageal cathepsin.

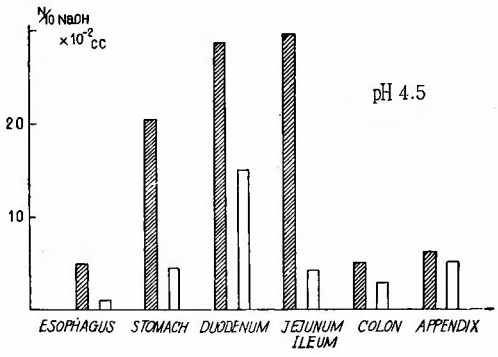

Fig. 8 Distribution of cathepsin in the digestive tracts of dogs.
▨ : Catheptic activities comprised in the mucous layers.
▭ : Catheptic activities comprised in the muscular or seromuscular layers.

Table 9 Catheptic activity in the necrotic area.
Acidity increase (N/10 NaOH c c)

Small Intestine		pH 4.0			
		Necrosis		Normal	
	Time	24	48	24	48
M	Upper	0.09	0.08	(0.65)	(0.40)
	Lower	0.36	0.43		
S	Upper	−0.13	0.15	(0.02)	(0.02)
	Lower	0.39	0.91		

will be amply presumed in the light of the results of the perfusion experiments by means of bile, pancreatic juice, trypsin and pepsin in the esophagus of the experimental animals, carried out by Cross, F. S. & O. H. Wangensteen, Takatsuki, one of our co-workers, and more recently, by Kato. Also, bacterial infection, as maintained by Jackson, C., and Mosher, H. P., may not be overlooked as a factor causing reflux esophagitis.

Chapter 1. Effect of Bile and Biliary Acid on Trypsin Digestion.

Section 1. Measurement of Trypsin Activity by means of Masuda's Modification of Fuld-Gross's Method.

When measuring the effect of bile on trypsin digestion by means of the formol-titration, determination of the neutral point was rendered difficult due to the coloring of bile, and this method, in the main, was adopted.

(1) Method of Experiment:

A pipette for measuring the descending speed of red blood corpuscles was used. From its end, 15% gelatine solution (pH 6.5～7.5) was injected, which was cooled and condensed, and by using a small pipette, the tested liquid [trypsin + buffer

solution + physiological salt solution, trypsin + buffer solution + biles of various densities (biliary acid)] was piled up in layers to the height of the column of 10 mm which was then placed in a 24°C incubator, and 48 hours afterwards, the length of the liquefied gelatine was measured. As control, sterilized distilled water, biles of various densities and buffer solution were piled in layers respectively. At the time of cooling, the surface of gelatine was seen to contract and sink. When a liquid not accompanied with a function of decomposing protein is piled in layers to this, the gelatine will expand and the affected portion will become higher by 2~3 mm. Therefore, in actually undertaking measurements, the expanded surface of gelatine was taken as zero point, with the length of the liquefied portion fixed at X mm, and the length of the expanded portion at Y mm, a formula (X + Y) mm was adopted as the degree of decomposition of protein. For trypsin, "Trypsilin" (mochida) was used.

(2) Experimental Result:

(i) Relation between the degree of decomposition of protein and the density of trypsin (Table 10, Figure 9).

The degree of decomposition of protein was proportional to the logarithm of the density of trypsin.

(ii) Relation between the degree of decomposition of protein and pH (Table 11, Figure 10).

Table 10 Relation between the degree of decomposition of protein and the concentration of trypsin.

Concent. of trypsin \ Time hrs	24	48
10u/cc	2.5	5.4
100u/cc	4.5	8.1
500u/cc	4.9	10.2
1000u/cc	5.5	10.2
5000u/cc	7.5	12.7
10000u/cc	7.8	13.0
100000u/cc	7.3	13.0

Table 11 Relation between the degree of decomposition of protein and pH.

pH \ Time hrs	24	48	72
4.90	4.0	6.3	8.8
6.60	5.3	7.4	9.0
7.10	5.4	7.6	9.2
8.25	4.7	6.8	7.7

Trypsin 500u/c c

While it is reported that the optimal pH for trypsin activity lies between pH 8.0-9.0, the following experiment was undertaken in order to ascertain the optimal pH of trypsin used in the present experiment. The degree of decomposition of

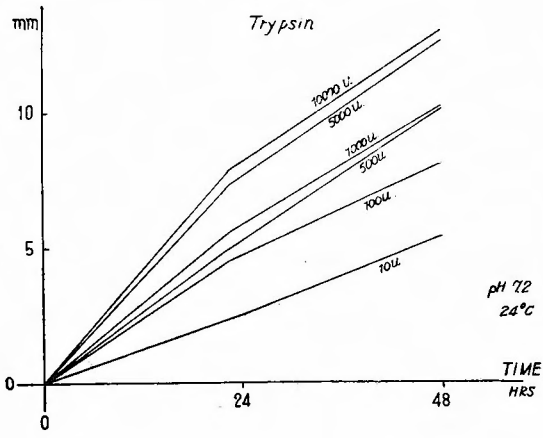

Fig. 9 Relation between the degree of decomposition of protein and the concentration of trypsin.

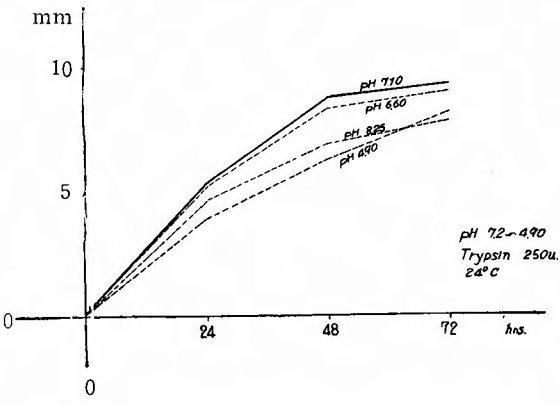

Fig. 10 Relation between the degree of decomposition of protein and pH.

protein indicated its largest value at pH 7. 10. This pH value was obtained by measuring the pH of the tested liquid with the method of glass electrode prior to the experiment.

(iii) Effect of bile on trypsin digestion (Table 12, Figure 11).

Table 12 Effect of bile on tryptic activity.

Bile \ pH	7.5	6.2
Control	7.1	6.9
0.1%	7.4	6.9
1 %	7.5	7.2
2 %		7.4
10 %	7.5	6.9
20 %	7.6	6.8
50 %	7.0	6.4
100 %	6.9	6.0

Trypsin 1000u/cc

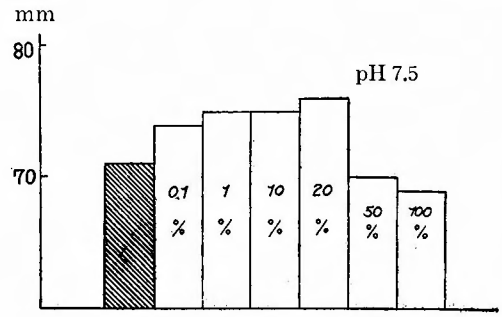

Fig. 11 Effect of bile on the tryptic activity (48hrs).

The bile was taken from the gall-bladder of healthy, adult mongrel dogs by means of abdominal incision and punction. Also, in order to avoid any individual difference, the biles taken from four dogs were mixed and diluted according to their respective percentage.

The tested liquid was composed of one part diluted bile and one part trypsin solution (1,000 u/c c), pH being placed at 7.5 and 6.2. According to the result thus obtained, it was known that the trypsin activity will be slightly stimulated by 10~20% bile in any pH.

(iv) Effect of biliary acid on trypsin digestion.

Moreover, the effect of biliary acid, the principal ingredient of bile, exerted on trypsin activity was examined.

Table 13 Effect of T. C. S. on tryptic activity.

	pH 6.2 (48 hrs)		
Height of superposed fluids	10	20	30
Control	6.8	6.9	7.0
M/1000 T. C. S.	7.4	7.5	
M/100 T. C. S.	7.3	7.6	
M/10 T. C. S.	6.8	6.7	

Trypsin 80u/cc

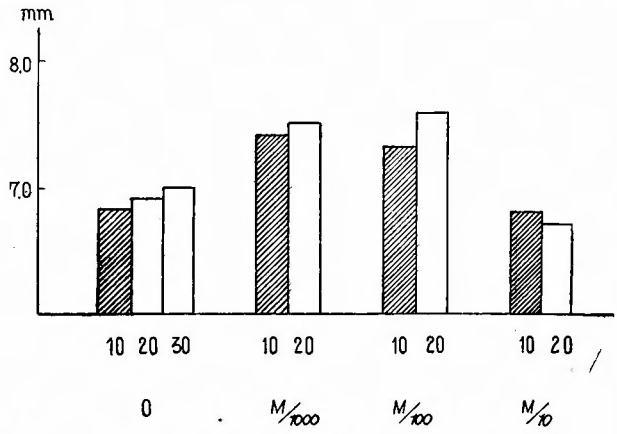

Fig. 12 Effect of T. C. S. on tryptic activity.

Table 14 Effect of T. C. S. on tryptic activity.

Time (hrs)	12	24	48
Control	1.8	2.8	5.2
M/10000 T. C. S.	2.0	3.6	5.7
M/1000 T. C. S.	2.0	3.8	5.9
M/100 T. C. S.	2.0	3.3	5.8
M/10 T. C. S.	1.7	2.9	5.1

Trypsin 30 u/cc

(a) Effect of Sodium taurocholate.

Sodium taurocholate (abbreviated as T. C. S. hereinafter) manufactured by the Nutritional Biochemical Corporation (U. S. A.) was used. The final density of trypsin of the tested liquid stood at 80 u/cc. Again, the degree of decomposition of protein, at pH 6.2 and pH 7.5, and with the height of the tested liquid column fixed at 10 mm, 20 mm and 30 mm, respectively, was examined. The results thus obtained are as shown in Tables 13 and 15 and in Figures 12 and 14. It was activated with M/100, M/1000 T. C. S., but, with M/10 T. C. S., it was inhibited.

Table 14 and Figure 13 shows the hourly change of the effect of T. C. S.

Table 15 Effect of T. C. S. and C. S. on tryptic activity (48 hrs).

pH		6.2	7.5
Control		6.2	7.8
T. C. S.	M/10000	6.7	8.1
	M/1000	6.3	7.9
	M/100	5.8	7.9
C. S.	M/10000	6.6	8.1
	M/1000	6.6	7.9
	M/100	6.6	7.9

Trypsin 50u/cc

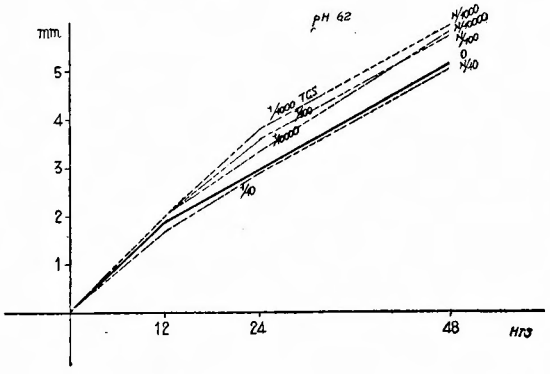

Fig. 13 Effect of biliary acid on tryptic activity.

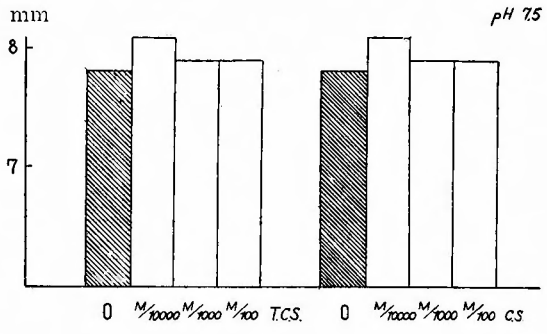

Fig. 14 Effect of T. C. S. on tryptic activity.

exerted on the decomposition of protein at pH 6.2 with the final density of the trypsin solution standing at 30 u/cc.

(b) Effect of Sodium Cholate.

For sodium cholate (abbreviated as C. S. hereinafter), the special class reagent manufactured by the Ishizu Seiyaku (Pharmaceutical Co.) was used. C. S. was also examined with a similar method. C. S., like T. C. S., was activated at the density of M/1000 and M/100, and inhibited at the density of M/10.

(c) Effect of Sodium Cholalate (Merk) (Figure 15).

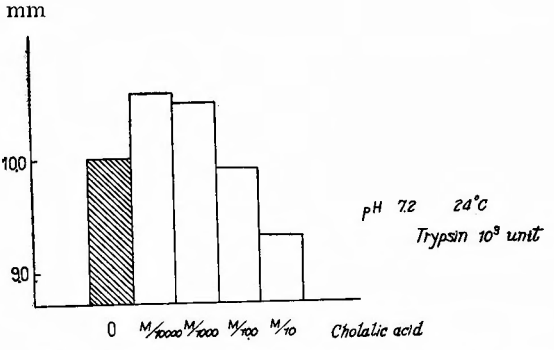

Fig. 15 Effect of biliary acid on tryptic activity.

Similar results, as in the two preceding experiments were obtained.

Section 2. Measurement of Trypsin Activity by Means of Formol-Titration.

(1) Experimental Material:

(i) Enzyme solution: As in the preceding section, trypsilin (mochida) was used, which was dissolved in a buffer solution at the rate of 0.4 mg/cc.

(ii) Substrate solution: 4% gelatine buffer solution.

(iii) Buffer solution: SOERENSEN's phosphate buffer solution (pH 6.5 and pH 7.2).

(2) Method of Experiment:

As the method of measuring the degree of decomposition of protein, a micro-burette was used, and by means of SOERENSEN's formol-titration, the increase of acidity was measured. As the main reaction mixture, 2.0 cc enzyme solution, 10.0 cc buffer solution, 10.0 cc substrate buffer solution, 2.0 cc bile or biliary acid solution or distilled water and 0.5 cc toluene were mixed, and after pH was rectified, 5.0 cc of the reaction mixture was taken with the pipette. Immediately after this, its acidity was titrated with N/10 NaOH solution, and tightly-plugged, and incubated for 24 hours, after which 5.0 cc of it was titrated, and the value, deducting the control value from the increased amount of acidity, was taken as the value of the decomposition of substrate by the enzyme solution. As control, the buffer solution, not carrying the substrate, was mixed with the enzyme solution, which was placed under the same condition with the main reaction mixture.

Table 16 Effect of bile and biliary acid on tryptic activity (Formol-titration).

pH 6.5			
Time (hrs)		24	48
Control		0.20	0.22
Bile	× 1	0.24	0.22
	× 10	0.22	0.23
	× 100	0.28	0.29
	×1000	0.26	0.26
T. C. S.	M/10	0.19	0.21
	M/100	0.21	0.25
	M/1000	0.28	0.29
	M/10000	0.21	0.24

Trypsin 0.4mg/cc

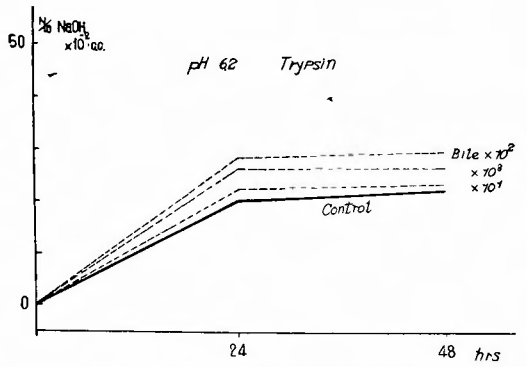

Fig. 16 Effect of bile on tryptic activity (Formol-titration).

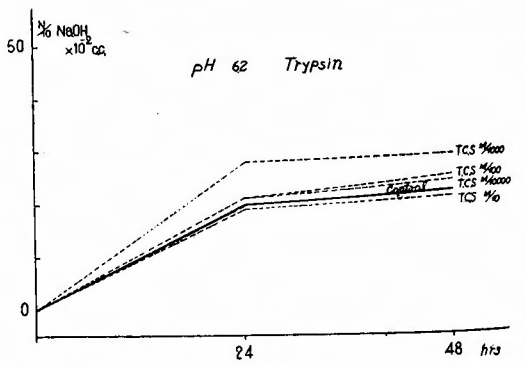

Fig. 17 Effect of T. C. S. on tryptic activity (Formol-titration).

(3) Experimental Results:

As shown in Table 16, Figures 16 and 17, the increase of acidity was greater as compared with the control, when bile and biliary acid were added. In other words, the trypsin activity was found to be activated by bile and biliary acid, although the density of the strongly activating biliary acid stood at M/1000.

Summary

As a result of examining the effect of bile and biliary acid exerted on trypsin digestion by means of MASUDA's modification of the FULD-GROSS's method and formol-titration, the results obtained were roughly similar, and the trypsin activity was found to be slightly activated, at pH 6.2 and 7.5, by 10% bile and M/100, M/1000 T. C. S. or C. S., but, at a higher density (M/10 T. C. S., C. S.), its activity was known to be inhibited (Table 17).

Table 17 Influences of bile and biliary acid on tryptic activity.

Method of determination		Fuld-Gross (modified)		Formol-titration
pH		7.5	6.2	7.2 (6.5)
Bile	0.1%	+	±	+
	1 %	+	+	+
	2 %		±	
	10 %	+	+	+
	20 %	+	±	
	50 %	−	−	
	100 %	−	−	
T. C. S.	M/10000	+		+
	M/1000	+	+	+
	M/100	+	+	+
	M/10	−	−	−
C. S.	M/10000	+		
	M/1000	+	+	
	M/100	+	+	
	M/10	±	−	
Cholalic acid (sodium salt)	M/10000	+	±	+
	M/1000	+	+	+
	M/100	±	+	−
	M/10	−	−	−

Activation (+) Inhibition (−)

Chapter 2. Relation Between Trypsin Activity and Protease Activity of Bacteria.

(1) Experimental Material:

(i) Enzyme Solution.

(a) Trypsin: Trypsilin (mochida) 100,000 unit, contained in vial, was dissolved in a buffer solution at the rate of 0.4 mg/cc.

(b) Dried bacterial enzyme (dried bacterial powder): Bacillus coli (Kyoto

University Bacteriological Department strains B. coli communior) and staphylococcus (F. D. A. 209-P strains and TERASHIMA strains) were cultured on a pH 7.0 ordinary bouillon culture medium for 24 hours at 37°C, and then were again cultured on a bacteria-multiplying agar-agar flat bottle for 24 hours at 37°C. The bacteria mass thus obtained were gathered with physiological salt solution, rinsed once with physiological salt solution, centrifugally separated, and then were treated three times with four-time-volume pure acetone, and were again treated two times with three-time-volume ether, after which they were dried in a 37°C incubator and preserved in a desiccator. 4 g of the dried bacterial powder thus obtained was mixed in 100 cc of 50% glycerine and thoroughly ground in a glass mortar into a homogenous suspension, which then was used as enzyme source.

(ii) Otherwise, the process undertaken was similar to what was described in Section 2.

(2) Experimental Method:

Similar to the preceding chapter, the increase of the acidity was measured by means of the formol-titration.

The reaction compositions were as follows:

(i) 2.0 cc of trypsin solution, or 2.0 cc of distilled water.
(ii) 2.0 cc of biliary acid solution or 2.0 cc of distilled water.
(iii) Buffer solution (SOERENSEN): (pH 7.5) 10.0 cc.
(iv) Substrate buffer solution (4% gelatine buffer solution) 10.0 cc.
(v) Toluene 0.5 cc.

The taking of control and the method of measurement were the same as in Section 2.

Table 18 Relation between tryptic activity and protease activity of bacteria. Acidity increase (N/10 NaOH c c) 24 hrs, pH 7.5.

Concent. or bac. enzyme (%)	0	0.2	0.3	0.4
Control	0.00	0.14	0.14	0.20
Trypsin	0.24	0.38	0.40	0.43

(3) Experimental Result (Table 18):

The degree of decomposition of protein corresponded to the sum of the trypsin activity and protease activity of bacteria. It was observed that the trypsin activity was not stimulated by adding a small amount of bacterial enzyme.

Chapter 3. Effect of Bile and Biliary Acid on Cathepsin Activity.

The distribution of cathepsin in various regions of the digestive tract has earlier been explained. The author has also undertaken an experiment on the effect of bile and biliary acid exerted on cathepsin activity.

(1) Experimental Method:

As was mentioned in Chapter 2, Part I, catheptase powder not-containing ereptase was made from the dog's esophageal membrane by means of the acetone-ether method. 10% suspension in distilled water of this powder was made into an

enzyme solution, and gelatine as a substrate, cysteine, as an activator, and bile or biliary acid were added, and by means of the formol-titration, the cathepsin activity was measured.

The reaction mixture were as follows:

(i) Enzyme solution 2.0 cc.
(ii) 0.05 M cysteine hydrochloride solution 2.0 cc.
(iii) Bile or biliary acid solution 2.0 cc.
(iv) Substrate buffer solution (4% gelatine buffer solution) 10.0 cc.
(v) Buffer solution (pH 4.5, Mc Ilvaine) 10.0 cc.
(vi) Toluene 0.5 cc.

The taking of the control and the method of measurement were made similar to those in Section 2.

Table 19 Effect of bile and biliary acid on catheptic activity.
Acidity increase (N/10 NaOH c c)

Time		24 hrs		48 hrs	
Activator (cystein)		none	added	none	added
Control		0.03	0.05	0.03	0.06
Bile	×2		0.03		0.04
	×10	0.00	0.04	0.02	0.04
	×100	0.00	0.03		0.03
	M/10		0.04		0.05
	M/100		0.03		0.04
	M/1000	0.02	0.05	0.02	0.05

(2) Experimental Result (Table 19, Figure 18) :

No activation of cathepsin activity by bile or biliary acid was observed. On the contrary, when one of these was added in a high density an inhibition of the activity was noted.

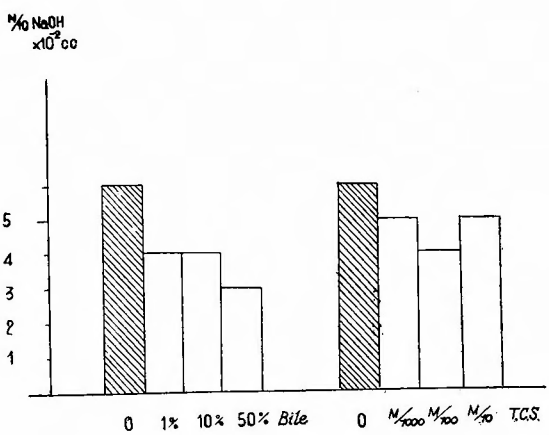

Fig. 18 Effects of bile and T. C. S. on catheptic activity.

Chapter 4. Effect of T. C. S. on the Ability of Hyrdochloric Acid to Penetrate into Stomach and Esophageal Membrane.

(1) Method of Experiment:

As shown in Figure 19, two bi-analogous glass cylinders were manufactured, the contact surface of which was circular 1 cm in diameter and which was provided with a turned-up brim. The radius of the base surface of these cylinders was 1 cm, while the height was 4 cm. The mucous membrane was taken

from the stomach and esophagus of healthy adult mongrel dogs, and with caution not to injure the surface of the mucous membrane, it was separated from the sub-mucous tissue.

Between the two cylinders, the stomach and the esophageal mucosa were extended respectively in each experiment, then fixed with a rubber band. At this time the surface of the mucous membrane was made to face a cylinder on the right side into which hydrochloric acid was to be poured. First, 10.0 cc of T. C. S. each was poured into the right and left cylinders, which were left for 30 minutes in a 37°C water tank (distilled water was placed in the contrast for similar operation), and this liquid was removed from the bottle mouth and the interior was rinsed with distilled water. After this, 10.0 cc of N/10 hydrochloric acid was placed in the right cylinder, while 10.0cc of distilled water was poured into the left cylinder with the contrast subjected to similar operation. The cylinders were again left for 30 minutes in a 37°C water tank, and the amount of Cl′ penetrating into the left cylinder was measured by means of the VOLHARD-HARVEY's method. 5.0 cc was taken out of the right cylinder, 20.0 cc of water and 20.0cc of N/10 $AgNO_3$ were added, and as an indicator, 2.0 cc of Ammonium iron sulfate $Fe(NH_4)(SO_4)_2 \cdot 12H_2O$ solution (acidified by adding nitric acid) was added, and was titrated with N/10 NH_4CNS for measuring the Cl′ amount.

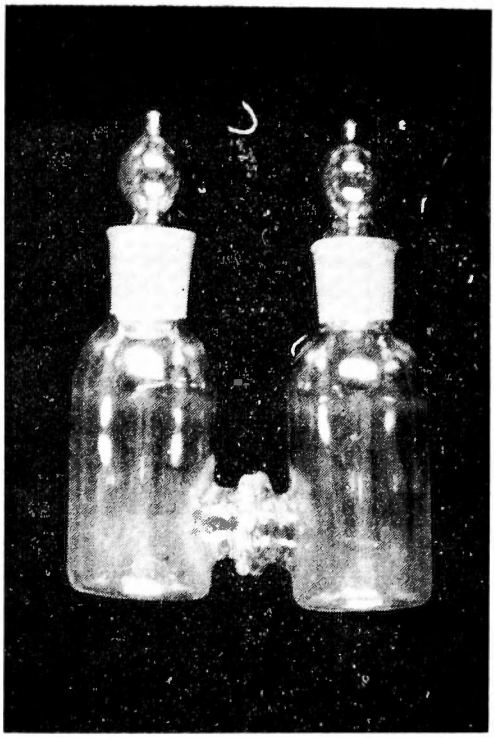

Fig. 19

(2) Experimental Result:

As shown in Figure 20, the normal esophageal mucous membrane, as compared with the normal stomach membrane, indicated a lower infiltration value of hydrochloric acid. It is considered in this connection that the thickness of the mucous membrane of dog's stomach and esophagus, respectively, has much to do with this. In case of treating with biliary acid, a slight stimulation of hydrochloric

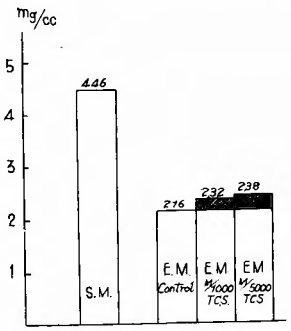

Fig. 20 Effect of T. C. S. on the ability of hydrochloric acid to penetrate into stomach and esophageal membrane.
S. M.: Mucous membrane of the stomach.
E. M.: Mucous membrane of the esophagus.

acid infiltration property of the esophageal membrane was noted.

DISCUSSION

Regarding the causes of reflux esophagitis, which is frequently complained of after a surgical operation on the esophagus, it is considered that besides the removal of the diaphragmatic pinchcock mechanism, which plays an important role in the prevention of such reflux, such factors as stomach atony and the stagnation of stomach contents, resulting from vagotomy, work in such a way that the contents of the stomach or intestines flow back into the esophagus, with the result that acid-pepsin digestion or trypsin digestion functions against the low-resistant esophageal membrane, thus resulting in reflux esophagitis. As a matter of fact, YAMAGUCHI maintains that, in such cases where hydrochloric acid or trypsin exists in the gastric juice, esophagitis is liable to happen. As asserted by WANGENSTEEN, O. H. and others, the extraordinary low resistance of esophageal membrane protein against pepsin or trypsin digestion plays an important role in causing esophagitis. This fact was also quantitatively proved by TAKATSUKI, one of our co-workers, in measuring the resistance of purified mucous protein of esophagus, stomach, small intestines and colon by means of the modification of KLEINMANN's nephelometry. However, that such cyclized molecule of protein as that found in the living tissues hardly receives trypsin digestion, is well known by results of the application of trypsin drugs in surgical causes or by KIRCHHEIM's experimental result, and also, by a systematic study carried out with the use of synthetic peptide of BERGMANN, M. and others. BARANOFSKY, I. D. and O. H. WANGENSTEEN indicated that, when a hindrance in circulation due to venous stagnation exists in the mucous membrane of the esophagus, stomach and duodenum, these membranes will become more sensitive to the digestion of the gastric juice. Accordingly, it is hardly considered that with only pepsin or trypsin, normal living esophageal membrane will receive digestion. As factors facilitating the initiation of pepsin or trypsin digestion, such factors as activation of cathepsin based on the action of acid or, interrupted circulation, bile (particularly biliary acid) and bacteria may be considered.

The studies on cathepsin, ever since WILLSTÄTTER, were mainly done on liver. According to WALDSCHMIDT and LEITZ, cathepsin is non-activated in normal tissues but will be activated by the stoppage of the living phenomenon of the tissues, leading to autolysis. KANZAKI maintains that cathepsin in a living body, is perfectly active, and, under the effect of the environment according to the living phenomenon, functions either decomposingly or synthetically. Furthermore, so far as the physical and chemical nature of the environment stays within a definite scope, it does not function decomposingly and, being apparently inactive, will function decomposingly in an abnormal condition, beyond the normal scope.

The study concerning the distribution of cathepsin in a living body, has been comparatively few. ONOYAMA reported that, in the case of a toad, it is aligned in the order of kidney, liver, intestines, lung, stomach, myocardium and the skeletal muscle. On the other hand, UCHINO undertook similar studies on fish.

First of all, the author quantitatively studied the amount of cathepsin distributed in various regions of the digestive tract. The optimal pH of esophageal cathepsin was 4.5～5.0, and he made it clear that it is provided with a considerably extensive operating pH area, and noted that it receives an activating effect by cysteine. At any region of the digestive tract, more cathepsin was found to exist in the mucous layer than in the muscular layer or the tunica serosa, while it was distributed in the order of small intestines, stomach, colon and esophagus, there being no tendency that especially more was distributed on the esophageal wall. Such distribution by layer of cathepsin was also confirmed by collaborating researcher MAJIMA in his histochemical examination of cathepsin in the digestive tract, which well coincides with the progress of esophagitis.

Reflux esophagitis is especially liable to be caused in the region where esophagus is isolated, i. e. in the vicinity of the region where it is anastomosed to stomach, duodenum or jejunum. It goes without saying that the contents of the digestive tract are liable to flow back near the anastomosed region. The esophageal cathepsin will be activated by interruption of blood circulation due to devascularization, inclination to the reduction side of local oxidized reduced electric potential and the lowering of local pH, as a result of which, it is considered that autolysis and peptic esophagitis will be brought about. So with this in view, change in the amount of blood circulation resulting from the isolation of esophagus was examined. An experiment of isolation of the entire length of thoracic esophagus of experimental dogs was carried out. But, contrary to the initial prediction, development of necrosis in esophagus was not noticed. The causes of the difficulty with which necrosis is caused as a result of isolation operation in esophagus will probably consist of the following three factors:

(1) Because in esophagus, the blood vessel network beneath the mucosa is closely anastomosed, isolation of esophagus will not easily cause a distinct interruption of blood circulation.

(2) In esophagus, the amount of cathepsin distributed is extremely small as compared with other parts of the digestive tract.

(3) Esophagus functionally consumes little oxygen of the tissues, and therefore, it can stand blood interruption.

The result obtained in the author's measurement of the amount of blood circulation in the normal esophagus conducted with the use of P^{32} labeled erythrocytes, approximately coincided with the results obtained in IPPONSUGI's experiment and OGAI's experiment on measurement of the amount of quicksilver distributed and the turbidity of the esophageal tissue by means of the injection of corrosive sublimate. In the lower part of thoracic esophagus and in the region immediately above the esophageal hiatus of the diaphragm, a region was found where the blood vessels and capillaries are poorly anastomosed. Especially on the right-side wall, the density of the blood vessels was seen to be low. As pointed out by collaborator TAKATSUKI, this region coincides also with the region where experimental reflux esophagitis is liable to be caused, and, in this sense, this observation should be considered to be

highly interesting.

On the other hand, the amount of circulating blood in the esophagus, as a result of the esophageal isolation, registered a further decrease as a whole, while maintaining their respective differences in the normal amount of circulation. In the region where circulation was in the worst condition, it reached a degree lower than 50% of the contrast. Even in this region, no necrosis was observed to be caused. Accordingly, it should be assumed that, in esophagus, autolysis or necrosis will hardly be caused even with the reduced amount of blood circulation because the amount of cathepsin distributed here is smaller as compared with other regions of the digestive tract. On the other hand, this fact apparently indicates that, in esophagus at least, even though cathepsin plays an important part as the first phase of digesting the living mucosa protein, that alone is insufficient. This cathepsin activity coupled with various factors in the contents of reflux, especially pepsin or trypsin, will enable it to strengthen the digestion of the mucous membrane.

When bile or pancreatic juice, trypsin and pepsin are injected experimentally into the esophageal membrane, an acute case of esophagitis will occur. This has been clarified by studies carried out by Cross, F. S. & O. H. Wangensteen, Takatsuki and Kato. Generally, the change on the esophageal wall due to bile and pancreatic juice is most violent, while a strong change will be noticed also with bile or biliary acid solution. It is reported however, that a slight change will occur with only pancreatic juice. In the simple method of esophagojejunostomy, bile will contact esophagus in a considerable density, which will cause esophagitis, frequently delaying the wound healing at the suture line.

Regarding the significance of bile which is considered to play a most important part in causing esophagitis, the author conducted the aforementioned experiment.

On the significance of bile against trypsin activity, Vonk, H. J. and others hold that bile, in a comparatively high density, will activate trypsin activity at pH 6.2, and will inhibit it at pH 8.0. In the experiment conducted by the author, bile or biliary acid at pH 6.2 and 7.5 evidently activated trypsin activity. And the density of biliary acid thus activating, coincides with the value expected to exist in the reflux contents.

Regarding the tissue injuring effect of bile, it is known that cellular fusion will occur due to the strong surface tension reducing activity and virulence of biliary acid. Rywosh, D. has pointed out the poisoning effect of biliary acid base against various tissues, while Fujioka and Yoshikawa affirmed that gastric ulcer will be caused by administration through the mouth or injection in stomach of biliary acid.

Tamesue undertook an experiment on the effect of sodium taurocholate exerted on the infiltration into the stomach membrane of Cl', and noted that infiltration into the stomach membrane is stimulated by biliary acid. The author, likewise, carried out an experiment on the esophageal mucosa, and could obtain approximately similar results.

Judging from these facts, the part of bile in connection with the occurrence of esophagitis may be considered to consist in the following:

(1) Bile activates trypsin activity;

(2) By the activity of bile to stimulate infiltration into the esophageal membrane, the esophageal mucosa, becomes liable to receive the primary tissue injury of acid or alkali, causing degeneration of protein and change in pH of the enzyme activity environment and come to lose resistance to protease activity.

In surgical esophageal and stomach diseases, bacillus coli and Cl. WELCHII exist to a high degree in the stomach, when a large quantity of gastric juice stagnates after the operation. This has already been reported by KUMAGAI and ENDO.

At the time of esophagogastrostomy, or -jejunostomy, protease and lecithinase activity of bacteria existing in the anastomosed region should, of course, be numbered among the factors causing esophagitis.

However, between bacterial protease activity and trypsin activity, no specific activating or multiplying activity was observed. Namely, the activity of both with different peculiarities against the substrate was represented as an algebraic sum.

Regarding the effect of bile exerted on the esophageal cathepsin, the experimental results obtained by ISHIKAWA, TAMESUE and NAKATA agreed in affirming that cathepsin activity will be inhibited by bile and biliary acid.

The foregoing may be summerized as follows: As for the causes of reflux esophagitis, the part played by such external factors as acid (alkali), pepsin, trypsin, bile and bacteria which function activatingly or cooperatively against their activity, or which make it easy to receive their respective activity, should be emphasized, while, as internal factors, the abnormally low resistance of the esophageal mucous protein against the digestive enzyme and the activity of esophageal cathepsin should probably be taken into consideration.

CONCLUSION

In order to clarify the causes of reflux esophagitis, a chemical examination was carried out regarding such internal factors as esophageal cathepsin and interruption of the esophageal circulation, and such external factors as bile and bacteria which are mixed in the reflux contents. The following results were obtained:

(1) An especially large quantity of esophageal cathepsin was found in the mucous layer. But, compared with other regions of the digestive tract, its amount distributed was abnormally small. This offers an explanation for the fact that, even with the isolation of the entire length of the thoracic esophagus in experimental dogs, necrosis was not caused, and moreover, indicates that for causing reflux esophagitis, participation of various external factors besides the esophageal cathepsin activity, autotissue-protein splitting enzyme is required.

(2) Bile, and especially biliary acid, will slightly activate trypsin activity with the density in the contents of the intestinal tract, and again, they also by themselves, function to increase the infiltrating ability of hydrochloric acid into the esophageal membrane, playing an important part in causing reflux esophagitis.

(3) The bacterial protease functioned cooperatively against trypsin digestive activity, but no tendency was noted that even a microscopic amount of the former,

pecially activated the latter.

The author wishes to thank Dr. KOICHI ISHIGAMI, the instructor of our clinic, for s many valuable suggestions and criticism throughout the present investigation.

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Fig. 1 Esophageal blood vessels. Radiopaque mass was injected into the thoracic aorta.

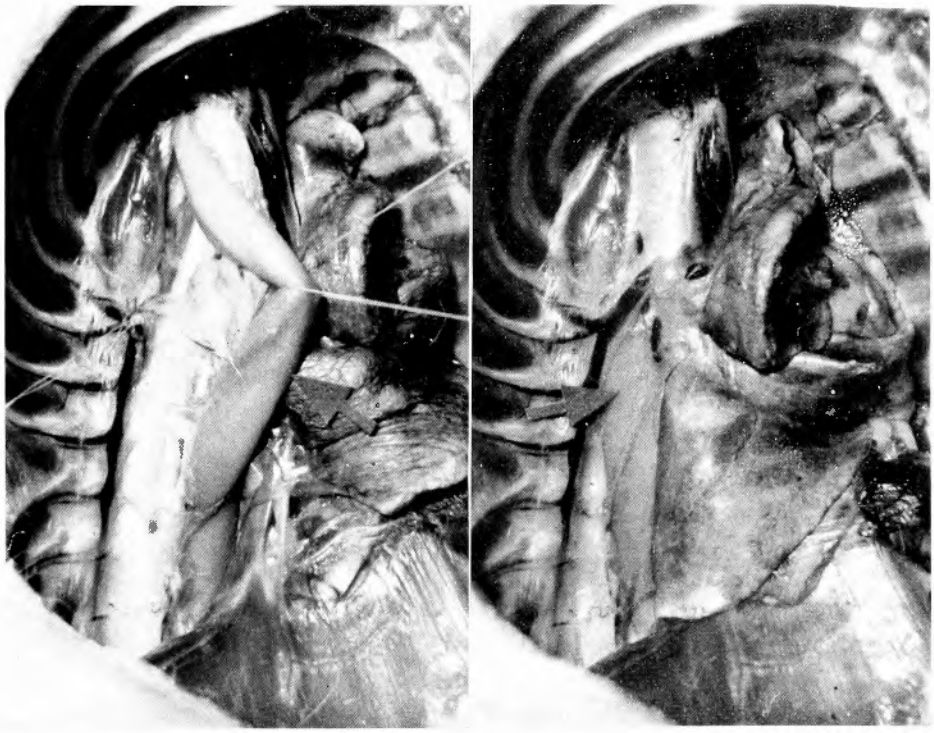

Fig. 2 Devascularization of the thoracic esophagus. The entire length of the thoracic esophagus was completely isolated from the adjacent tissues.

Fig. 3 Dog No. 28, on the 5th postoperative day. No necrosis was observed in any part of the esophagus.

Fig. 4 Microphotograph (×100) of section of the esophagus. No pathological findings were observed.

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和文抄録

逆流性食道炎の成因に関する実験的研究，とくに食道カテプシン，胆汁および細菌感染の意義について

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食道，胃噴門部の切除吻合術後に屢々みられる逆流性食道炎の成因に関しては，さきに共同研究者高槻が実証したように，食道粘膜蛋白質がペプシンまたはトリプシン消化に対して異常に低い抵抗性を示しているということが大きな役割を演じていることは明らかであるが，この他に生活食道粘膜蛋白質のような環状蛋白質に働いて，トリプシンまたはペプシン消化を受け易くする因子として，酸，アルカリ，血行障害にもとずくカテプシンの賦活，逆流内容に含まれる胆汁（とくに胆汁酸），細菌等の因子を考慮すべきものである．著者は，食道をはじめとする消化管各部位におけるカテプシンの分布量を層別に，フオルモール滴定法を用いて定量し，食道カテプシンは，とくに粘膜層に多く分布しているが，消化管の他の部位における分布量と比較すると，異常に少い値を示すことを証明した．このことは実験犬の胸腔内食道を全長にわたつて遊離しても壊死を発生することがなかつたことに対して，一つの説明をあたえるものであり，また逆流性食道炎の発生には，食道に存する自己蛋白分解酵素カテプシン以外に種々の外来性因子の関与を必要とすることを示している．

胆汁とくに胆汁酸は，腸管内容における濃度においては，トリプシン作用を軽度に賦活し，またそれ自身単独でも，食道粘膜の塩酸滲透性を増大せしめる作用を有し，逆流性食道炎の発生に重要な役割を演ずるものである．

細菌プロテアーゼは，トリプシン消化作用に対して協同的に作用したが，前者の微量が後者をとくに賦活するという傾向は認められなかつた．